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Intrabody construction and expression III:
Engineering hyperstable V_H domains

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Abstract

The folding of immunoglobulin domains requires the formation of a conserved structural disulfide. Therefore, as a general rule, they cannot be functionally expressed in the reducing environment of the cellular cytoplasm. We have previously reported that stability engineering can lead to the cytoplasmic expression of functional immunoglobulin V_L domains. Here we apply rational stability engineering by consensus sequence analysis to V_H domains. Isolated V_H domains tend to aggregate more easily than V_L domains; they do not refold quantitatively and are generally more difficult to handle in vitro. To overcome these problems, we successfully predicted and experimentally verified several stabilizing point mutations in the V_H domain of a designed, catalytic Fv fragment. The effect of single mutations was additive, and they could be combined in a prototype domain with significantly improved stability against chemical denaturation and a 20-fold increased half time of irreversible thermal denaturation, at physiological temperature. This stabilized, isolated V_H domain could be expressed solubly in the reducing cellular cytoplasm of *Escherichia coli*, at a yield of approximately 1.2 mg/L of shake flask culture. It remains fully functional, as evidenced by the successful reconstitution of an esterolytic Fv fragment with the V_L domain. This success provides further evidence that consensus sequence engineering is a rational, plannable route to the construction of intrabodies.

Keywords: intrabodies; protein engineering; protein stability; recombinant expression; VH domain

Because the folding of immunoglobulin domains almost invariably requires the formation of a conserved structural disulfide bond [between Cys22 (the numbers of the individual amino acid residues are according to the Kabat sequence database; Kabat et al., 1992) and Cys92 in V_H domains], their soluble expression in the reducing cellular cytoplasm has failed in general (Glockshuber et al., 1992). Few exceptions have been reported, notably scFvs against HIV proteins (Maciejewski et al., 1995; Wu et al., 1996; Rondon & Marasco, 1997), but low stability, low expression rates, and unpredictable behavior currently limit these perspectives (Gargano & Cattaneo, 1997; Marasco, 1997), and most of these scFvs still form insoluble aggregates (Cattaneo & Biocca, 1999). Elegant screening methods and random mutagenesis have been employed in two cases, to evolve a levan-binding scFv intrabody (functional cytoplasmatic antibody) and an anti-β-galactosidase scFv intrabody (Martineau et al., 1998; Proba et al., 1998). Unfortunately, it

is not clear how these approaches can be generalized, because they have either depended on a specific immunoglobulin framework, naturally lacking a disulfide bridge, or a metabolically selectable ligand binding activity.

We have previously established a strategy to predict stabilizing mutations in immunoglobulin domains (Steipe et al., 1994). It is based on engineering consensus sequences, compiled in a freely accessible database (Steipe, 1998). In a previous manuscript (Ohage & Steipe, 1999), we have analyzed whether stability engineering can overcome the limiting steps in the soluble cytoplasmic expression of V_L domains. This was indeed the case, and based on this success, loop grafting onto a consensus framework has allowed the construction of a functional, catalytic intrabody Fv fragment (Ohage et al., 1999). Its paratopes were derived from 17E8 (Zhou et al., 1994), a well-characterized esterolytic antibody. Because the hapten is not normally present in the cell, complex formation does not contribute to domain stabilization.

The V_H domain used in these experiments was successfully co-expressed in the cytoplasm with a stabilized V_L. But other than using a natural framework sequence close to the consensus, this intrabody V_H domain required no further optimization after grafting the residues involved in substrate binding onto the framework. Thus, the possibility arises that we may have serendipitously combined the framework with new CDR loops in a favorable way, which might be special to the protein we had constructed. It re-

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Abbreviations: β-ME, β-mercaptoethanol; CDR, complementarity determining region; Fv, heterodimer containing V_L and V_H; scFv, single-chain Fv fragment; GdmCl, guanidinium chloride; ΔG_F, free energy of folding; IMAC, immobilized metallion affinity chromatography; NFMP, N-formyl methionine-phenylester; PBS, phosphate-buffered saline; V_L and V_H, variable domains of immunoglobulin light and heavy chains, respectively.

mains to be shown whether consensus sequence engineering can be applied to V_H domains, to improve their folding and stability for the construction of intrabodies also in less favorable cases.

Finally, it has to be investigated whether mutations introduced for stability engineering affect the activity of the catalytic intrabody Fv fragment from which the V_H domain IcaH-01 originally was derived.

Results

Determination of stability changes for oxidized domains

The mutations that were predicted to stabilize the reference domain IcaH-01, the V_H domain of a synthetic catalytic antibody (Ohage et al., 1999), are shown in Table 1. They replace residues present in the original natural framework with ones that are more frequent in the sequence database (Steipe, 1998). We have prepared isolated V_H domains to determine the thermodynamic effects of our mutations, but the refolding of isolated V_H domains was not fully reversible. In the absence of an equilibrium, the free energy of folding cannot be determined. In contrast to isolated V_H domains, unfolding was fully reversible in the presence of equimolar amounts of the V_L domain Ica-L11. Thus equilibrium unfolding transition curves were determined for heterodimeric Fv fragments at identical protein concentrations. The individual transitions for the V_H and V_L domains are well separated, allowing subtraction of the V_L signal to obtain the unfolding curves for V_H domains in the presence of folded V_L (Fig. 1). Under these conditions, resistance to denaturation is influenced by the formation of heterodimers and becomes concentration dependent. Thus, no quantitative interpretation of the transition curves was attempted; rather, the relative effect of the mutations on thermodynamic stability was estimated by the shifts in denaturation midpoints, relative to IcaH-01. This is valid to the extent that mutations do not significantly affect the association of dimers. Indeed, all of the mutations but one are distant from the domain interface, and the effect of mutating this one residue, R^{43}, is not qualitatively different from the other mutations, for heterodimers, as well as for isolated domains (see below).

Fig. 1. Equilibrium transition curves for the Ica-Fv fragment and the isolated IcaL-11 domain 2 μM each, pH 7.4, 20 °C (see details in Materia and methods). The V_L data were shifted by 168 units relative to the V_H, b shown at the same scale. Two well-separated transitions are observed.

Of the seven mutations we tested, four significantly shifted th transition midpoints to higher denaturant concentrations (Table 1) A fifth mutation ($P^{07}S$) had no significant effect on the transitio midpoint, but showed an increased cooperativity of folding. Non of the consensus mutations were severely destabilizing.

Combination of mutations

Because the mutations are located throughout the V_H domain, they are expected to be independent, and their stabilizing effects should be simply additive. We, thus, set out to construct a hyperstable prototype V_H domain by successively combining individual point mutations (Table 2). This strategy was indeed successful. Although each single mutation has only a small effect on the equilibrium unfolding transition midpoint, the effects are additive, and in combination, they approximate the sum of the individual effects (Fig. 2). The mutation $P^{07}S$ is an exception, because it was found to be significantly more effective in the context of IcaH-401 than in the context of IcaH-01.

Thermal stability

Because of the presence of interacting V_L domains, the transition curves obtained for V_H cannot be interpreted in terms of the thermodynamic stability of isolated domains. The observed increase of

Table 1. *Prediction of point mutations and experimentally determined resistance to denaturation for single mutations in IcaH-01*

Mutation[a]	Native residue and frequency (%)[b]	Consensus residue and frequency (%)[b]	$\Delta[GdmCl]_{50}$ (M)[c]
$K^{03}Q$	K (19.6)	Q (67.5)	−0.03
$P^{07}S$	P (8.6)	S (87.8)	0.01
$A^{16}G$	A (26.2)	G (35.2)	0.1
$R^{43}Q$	R (6.1)	Q (26.2)	0.04
$I^{58}T$	I (10.2)	T (77.1)	0.08
$P^{74}S$	P (4.9)	S (77.4)	0.1

[a]Mutations chosen to be combined in a hyperstable prototype V_H domain are shown in bold.

[b]Amino acid frequency distributions taken from Steipe (1998). The combined list of V_H domains was used.

[c]Shifts in concentration of denaturant at which 50% of the folded domain fluorescence signal are lost, relative to IcaH-01.
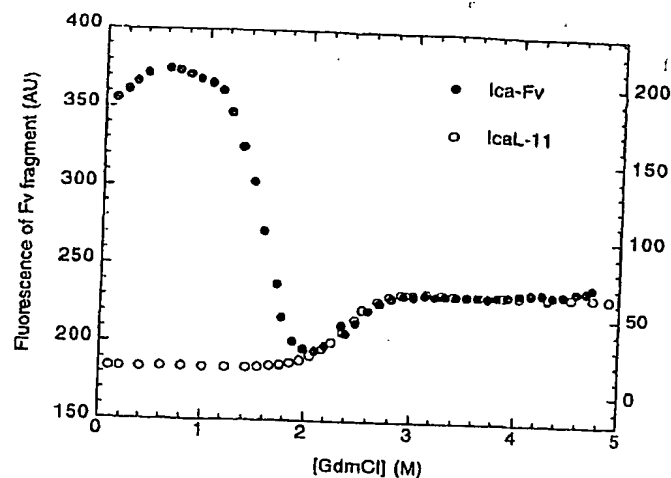
Table 2. *Combination of single point mutations*

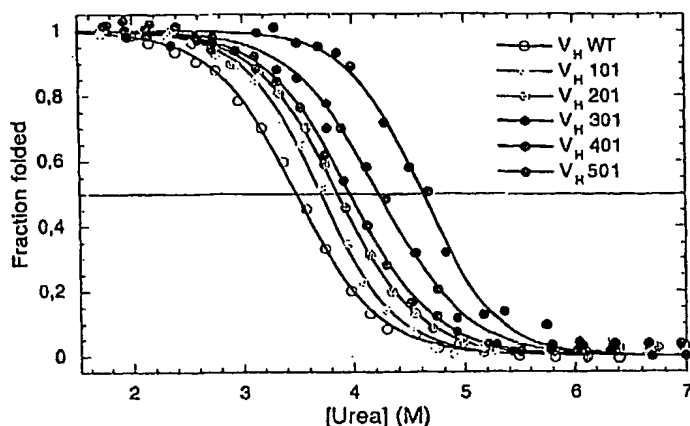| Protein	Mutations
IcaH-01	—
IcaH-101	$A^{16}G$
IcaH-201	$A^{16}G$, $I^{58}T$
IcaH-301	$A^{16}G$, $I^{58}T$, $R^{43}Q$
IcaH-401	$A^{16}G$, $I^{58}T$, $R^{43}Q$, $P^{75}S$
IcaH-501	$A^{16}G$, $I^{58}T$, $R^{43}Q$, $P^{75}S$, $P^{07}S$

Fig. 2. Unfolding equilibrium transition curves for IcaH-01 and five stabilized mutants in the presence of equimolar concentrations of the V_L domain IcaL-11; protein concentration of 2 μM, pH 7.4, 20 °C (see details in Materials and methods). Each successive stabilizing mutation shifts the denaturation midpoint to higher denaturant concentrations. To guide the eye, transition curves were normalized and curve fits drawn with a least-squares parametric fit, assuming a two-state transition of a monomeric domain. If this model were correct (ignoring the interactions with the V_L domain), the free energy of folding would be ~22 kJ mol^{-1} for IcaH-01, and ~34 kJ mol^{-1} for IcaH-501.

resistance to chemical denaturation is ambiguous with respect to stability changes of the V_H domains or the stabilization of heterodimers. Therefore, we investigated whether irreversible thermal denaturation of isolated V_H domains would provide supporting evidence for improved stability. Irreversible denaturation presumably does not measure the thermodynamic stability, ΔG_{fold}, but the activation free energy for unfolding, ΔG_{unfold}. This does not require an equilibrium, and the measurement can be performed with isolated V_H domains. We observe a good qualitative correlation between increased tolerance of denaturant under equilibrium conditions and the rate of irreversible denaturation (Fig. 3). This increase in thermostability can be quantified by determining the time, respectively temperature, at which 50% of the protein remains soluble (Table 3). The thermostabilization we have achieved
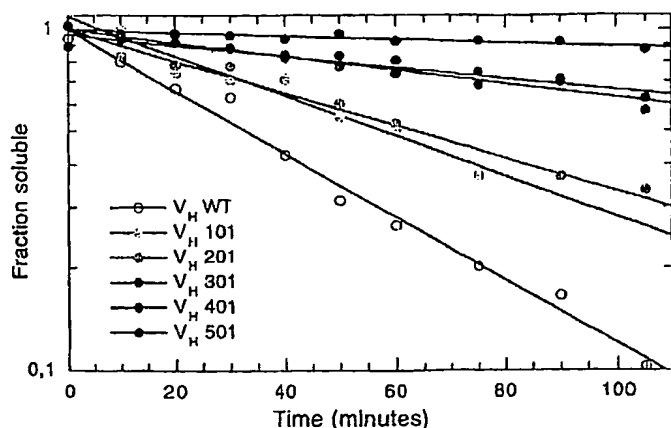

Fig. 3. Irreversible thermal denaturation of IcaH-01 and successively stabilized domains; protein concentration of 7.5 μM, pH 7.4, 100 mM β-ME (see details in Materials and methods).

Table 3. *Fifty percent survival of successively stabilized V_H domains*

Protein	t_{50}[a] (min)	$T_{50} \pm 1$ °C[b] (°C)	$[urea]_{50}$ (M)[c]
IcaH-01	31.2	36.4	3.63
IcaH-101	51.2	38.1	3.85
IcaH-201	63.2	39.8	3.88
IcaH-301	149	40.1	4.11
IcaH-401	184	41.4	4.34
IcaH-501	606	42.5	4.62

[a]t_{50} is the time after which 50% of the protein has become insoluble at 38 °C.

[b]T_{50} is the temperature at which 50% of the protein has become insoluble after 1 h of incubation. The values were obtained from a linear least-squares fit to the raw data from the temperature regions where aggregation was observed.

[c]$[urea]_{50}$ concentration of denaturant at which 50% of the folded domain fluorescence signal is lost.

with our approach of rational stability engineering is quite significant. Survival time at physiological temperature (38 °C) is increased 20-fold for IcaH-501, relative to IcaH-01. Accordingly, we observe reduced aggregation in equilibrium transition experiments for the isolated V_H domains (not shown).

Refolding and stability of reduced V_H domains

From comparing V_H transition curves, we estimate that the true increase in folding free energy is of the same order as the predicted effect of the structural disulfide bridge on the domain stability (Pace et al., 1988; Ohage & Steipe, 1999). We thus determined whether IcaH-501 could be reversibly unfolded in the presence of GdmCl under reducing conditions (Fig. 4). Transition curves for
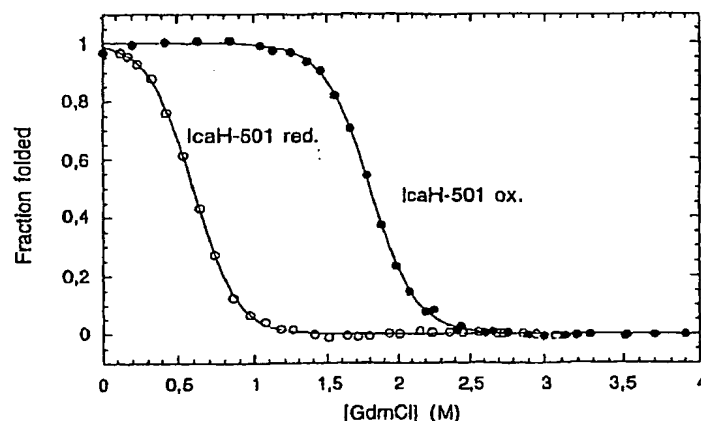

Fig. 4. Equilibrium unfolding transitions for oxidized and reduced IcaH-501 in the context of the Fv fragment; protein concentration of 2 μM, pH 7.4, 20 °C (see details in Materials and methods). To calculate curve fits, a two-state transition of a monomeric domain was assumed. This allows us to estimate the contribution of a disulfide bridge to be on the order of −15 kJ/mol, which is approximately the same value we have determined previously for V_L domains (Ohage & Steipe, 1999), and which is predicted from statistical mechanics, assuming the disulfide bridge affects exclusively the entropy of the unfolded state (Pace et al., 1988).

the heterodimeric Fv fragment again showed two well-separated, fully reversible transitions, even though the stability of reduced IcaH-501 is only marginal, and a small population of unfolded domains appears to be present even in the absence of denaturant. The V_H transition midpoint is shifted to a lower denaturant concentration by approximately 1.3 M of GdmCl, and by comparison with Figure 1, it can be estimated that the loss of the structural disulfide bond will almost fully unfold the IcaH-01 domain.

Successful cytoplasmic expression of isolated V_H domain

Finally, the direct cytoplasmic expression of IcaH-μ01 (the prefix "μ" refers to the presence of the initiator methionine; see Ohage & Steipe, 1999) and IcaH-μ501 was attempted. After separation of soluble and insoluble fractions of the cell extracts, IcaH-μ501 appeared as a prominent band in the soluble fraction after SDS-PAGE and could be purified by IMAC. The yield of soluble protein was approximately 1.2 mg/L shake flask culture. In contrast, no expression could be detected for isolated IcaH-μ01 domains.

Catalytic activity of an Fv fragment containing the V_H IcaH-501 domain

To investigate whether the introduction of mutations for stabilizing the IcaH-01 domain had an influence on the hydrolytic activity of the catalytic Fv fragment from which the IcaH-01 domain originally was derived (Ohage et al., 1999); the hydrolysis rates for the hapten N-phenyl methionine ester of a cytoplasmatic Fv fragment containing either IcaH-01 or IcaH-501 were compared. The measurements were carried out in PBS (pH = 7.4) under reducing conditions (0.3 mM β-ME). The activity of both the Fv fragment containing IcaH-01 as well as IcaH-501 followed Michaelis–Menten kinetics. We determined a K_M of 299 μM, k_{cat} of 1.61 min^{-1} and k_{cat}/k_{uncat} of 3.5×10^3 for V_L IcaL-11 + IcaH-01 and K_M of 155 μM, k_{cat} of 1.34 min^{-1} and k_{cat}/k_{uncat} of 3.0×10^3 for V_L IcaL-11 + IcaH-501. Thus, there is only an insignificant influence of the introduced V_H-stabilizing mutations on the activity of our catalytic Fv fragment.

Discussion

We have shown that a rational approach to stability engineering, based on consensus sequence approximations, provides a plannable route to stable V_H frameworks. Although isolated V_H domains behave differently from V_L domains in that they are prone to aggregation and, in general, cannot be expressed in the absence of V_L domains, we have shown that successive stabilization can overcome such problems and increase resistance to equilibrium chemical denaturation as well as to irreversible thermal denaturation. In our most stable mutant IcaH-501, the half time of survival of irreversible thermal denaturation of the isolated domain was increased 20-fold at physiological temperature. The same domain is now tolerant to reduction of the structural disulfide bond and can refold under reducing conditions.

Consensus sequence engineering requires no special features or properties of the immunoglobulin domain. Thus, the individual mutations can be easily predicted by comparing target sequences with a freely accessible database (Steipe, 1998). Their effects are independent and additive to a good approximation and, because they are only weakly dependent on context, the approach is expected to be completely general. In contrast to random mutagenesis and screening procedures (Proba et al., 1995; Martineau et al.,

1998), no non-natural sequence motifs special to specific constellations of residues are introduced. In this respect, consensus frameworks, representing an average of the evolutionary background of immunoglobulin domains, may be especially well suited for loop grafting. It is one of the corollaries of the canonical sequence approximation, that while each individual domain sequence has only a low probability of occurring in the ensemble, all discrete consensus epitopes occur in the ensemble with a relatively large probability. Thus, the approach may even lead to synthetic immunoglobulin domains with a decreased allergenic potential.

An interesting application of isolated V_H domains emerges from the fact that they can serve as recognition modules by themselves. Even in natural antibodies, V_H usually contributes the majority of interactions with the ligand. Accordingly, the use of a natural V_H as a minimal recognition domain has been reported (Ward et al., 1989), but expression and handling are significantly more difficult than for V_L domains, and solubility problems have precluded their widespread application. Naturally occurring, soluble antibodies devoid of light chains have been discovered in *Camelidae* species (Hamers-Casterman et al., 1993), and it was shown that the relevant residues could be engineered into other antibody frameworks (Davies & Riechmann, 1996; Riechmann, 1996). This has spar' renewed interest in isolated V_H domains: due to their small size and convex binding site, they appear to be superior to Fv fragments in binding into the catalytic cleft of enzymes and, thus, form novel inhibitors (Martin et al., 1997; Lauwereys et al., 1998). Thus, hyperstable V_H domains that can fold in the absence of the structural disulfide bridge would have potential as functional single domain intrabodies.

Finally, the successful expression of a soluble, isolated V_H domain in the cytoplasm of *E. coli*, together with our previous success of expressing different isolated V_L intrabody domains (Ohage & Steipe, 1999) and a catalytic intrabody Fv fragment (Ohage et al., 1999), demonstrates that domain stabilization by consensus sequence engineering provides a plannable route to designed intrabodies. The fact that the stabilizing mutations did not intefere with the domain function underscores the value of our approach for the construction of a variety of functional, stabilized antibody domains and intrabodies, once the sequence of the original antibody is known.

Materials and methods

Sequences of proteins in this study

The construction of the reference V_H domain for this study (IcaH-01) has been described (Ohage et al., 1999). Ica-H01 was designed for a catalytic intrabody and comprises sequences from the framework of the murine anti-nitrophenyl antibody B1-8 (Bothwell et al., 1981) and the three CDR sequences of the esterolytic catalytic antibody 17E8 (Zhou et al., 1994). Fv fragments additionally comprise the IcaL-11 domain, a precursor of the designed, catalytic intrabody V_L domain IcaL-μ14 (Ohage et al., 1999). The sequence for IcaH-01 is ^{1}EVKLQQPGAE ^{11}LVKPGASVKL ^{21}SCKASGYTF<u>T</u> ^{31}DHA<u>I</u>HWVKQR ^{41}PGRGLEWIG<u>Y</u> ^{51}ISP GNG<u>DI</u>KYN ^{61}EKFKSKATLT ^{71}VDKPSSTAYM ^{81}QLSSLTSED SAVY ^{91}YCAR<u>S</u>-YYGSS ^{101}Y<u>V</u>DYWGQGTTLTV ^{111}SSHHH HHH (loop grafted residues that differ from the natural B1-8 sequence are underlined). The sequence of the V_L domain IcaL-11, which was used for co-expression with V_H, is ^{1}DIVMTQSPSS ^{11}LSVSLGERVT ^{21}ISCKASQDIK ^{31}KYIGWYQQKP ^{41}GQPPK

LLIHY [51]TSTLLPGVPD [61]RFSGSGSGTD [71]FTLTISSVQA [81]ED
LAVYYCLQ [91]YYNLRTFGA [101]GTKLELKHHH [111]HH.

Choice of mutations

Stabilizing mutations were predicted to approximate the V_H consensus sequence that was compiled from a database based on Kabat et al. (1992) and Steipe (1998). Oligonucleotide-directed mutagenesis was performed by standard methods using single-strand DNA prepared from an f1 origin of replication in the vector. All modified genes were sequenced.

Expression and purification

For periplasmic expression in *E. coli* JM 83 cells, genes of interest were fused to the secretion signal of alkaline phosphatase (phoA) for V_L and outer membrane protein A (ompA) for V_H in a plasmid based on pASK75 (Skerra, 1994). Due to the oxidizing properties of the periplasm, secretion leads to spontaneous formation of the central disulfide bridges once the signal is cleaved off, followed by productive folding (Skerra & Plückthun, 1988). The proteins were harvested from the periplasmic fraction and purified to homogeneity by immobilized metal ion affinity chromatography (IMAC) on an iminodiacetic acid matrix (Pharmacia, Uppsala, Sweden) charged with Zn^{2+}. To obtain isolated V_H domains, they were co-expressed with V_L domains that did not possess a histidine tag. The complexes were bound to the IMAC column, heterodimers were dissociated by denaturation with 6 M urea, and V_L domains were eluted. V_H domains were quantitatively renatured in situ by incubation with IMAC buffer (300 mM NaCl, 50 mM NaPO4, 10 mM Tris, 2 mM β-ME, pH 8.0) for 20 min and eluted as a single, pure band with 300 mM imidazole.

For the cytoplasmic expression of isolated V_H domains, the vector ptetVH was used, which was derived from pIcaFv20c (Ohage et al., 1999) by deleting the V_L gene. V_H genes were cloned into the vector that was transformed into BL21(DE3) cells. Bacterial culture (500 mL) was grown to an OD_{600} of 0.9, expression was induced with anhydro-tetracycline, and cells were incubated for a further 3 h at 20 °C. Approximately 2.5 g of cell pellet was harvested by centrifugation and resuspended in 5 mL of IMAC buffer. The resuspended cells were lysed in a vibratory mill and centrifugated at 18,000 relative centrifugal force (rcf) for 10 min. The supernatant was pooled, the pellet was resuspended again in 2.5 mL IMAC buffer, and treated as above. The collected supernatants were applied to a nitrilotriacetic acid column (Quiagen, Hilden, Germany), charged with Ni^{2+}. Columns were washed with IMAC buffer containing 30 mM imidazole, eluted with 300 mM imidazole, and analyzed by SDS-PAGE. The protein concentration was determined by detecting the OD_{280} of the reduced, denatured protein (ϵ_{VHred} = 29,870) .

Estimation of thermodynamic stability

Folding transition curves were determined by reversible chemical denaturation of 2-μM solutions of the Fv fragment (V_H + IcaL-11 mixed together from separate stocks for the desired concentrations) in phosphate-buffered saline solution (PBS, 4 mM KH2PO4, 16 mM Na2HPO4, 115 mM NaCl, pH 7.4) with variable concentrations of guanidinium chloride (GdmCl) or urea, after incubation overnight at 20 °C. Under these conditions, the unfolding transition is completely reversible, and unfolding and refolding curves are

identical within experimental error. The folding transition curves of reduced domains were determined after unfolding Fv fragments in 4 M GdmCl in the presence of 50 mM DTT to ensure complete reduction. Transition curves were determined from the fluorescence signal. λ_{ex} = 282 nm; λ_{em} = 323 nm for oxidized domains and λ_{em} = 335 nm for reduced domains.

Determination of thermal stability of isolated V_H domains

To investigate the activation barrier for unfolding with irreversible thermal denaturation, we incubated oxidized V_H domains at varying times and temperatures with reducing buffer. We had determined previously that oxidized V_H IcaH-01 does not aggregate perceptibly after more than 2 h at 20 °C under the reducing conditions described below. Evidently, the reduction of the disulfide bridge requires unfolding of the domain. Unfolded domains are rapidly reduced; this increase in entropy traps them in the unfolded state and aggregation ensues. This protocol ensures that the unfolding process is indeed irreversible. In contrast, under oxidizing conditions V_H IcaH-01 does not aggregate perceptibly, even at 50 °C.

Isolated V_H domains were brought to a concentration of 7.5 μM in PBS, 100 mM β-mercaptoethanol. To determine the time dependence of thermal denaturation, 50 μL aliquots were incubated at 38 °C for variable times, cooled on ice for 5 s, and centrifuged at 18,000 rcf, 4 °C, for 5 min. To 30 μL of the soluble supernatant, 10 μL of a BSA solution of 0.5 mg/mL was added as an internal standard. Samples were run on a 12.5% SDS-PAGE gel, stained with Sypro Red (FMC Bioproducts, Rockland, Maine), and the bands were quantified on a phosphorimager (Molecular Dynamics, Sunnyvale, California). To determine the temperature dependence of thermal denaturation, samples were incubated for 1 h at temperatures from 35 to 44 °C in steps of ~1 °C, and the remaining soluble material was quantified as above.

Catalytic activity

Hydrolysis velocities were determined at 20 °C by the increase of the A_{270}, due to phenol release after diluting different amounts of racemic N-formyl methionine-phenylester (NFMP) into stock solutions of reduced Fv fragment in PBS (pH = 7.4) containing 0.3 mM β-mercaptoethanol, compared to the background reaction. NFMP was used as a stock solution in DMSO. The exact concentration of unhydrolyzed ester in the DMSO stock was determined photometrically, after total hydrolysis in 0.2 N NaOH and divided by 2, because the catalyzed reaction is specific for the L-enantiomer (Guo et al., 1994), compared to water. The concentration of the Fv fragment was 5.5 μM.

Acknowledgments

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